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Award Number: W81XWH-11-1-0548

TITLE: Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer

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REPORT DATE: October 2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual Report	25September2012–24Septemb2013

4. TITLE AND SUBTITLE

Enhancing the Breadth and Efficacy of Therapeutic Vaccines for Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0548

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS

Beckman Research Institute of City of Hope
1500 East Duarte Rd.
Duarte, CA 91010

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Over the last 12 months of this award, we have focused on the optimization of various systems for the identification of tumor-reactive T cells. Our team has tested and optimized protocols for the isolation of several immune cell types, the generation of antigen-presenting cells, and the culturing of T cells. We have also optimized protocols and systems to screen T cells for reactivity to cancer cell line antigens and the cloning of T cells. We have began screening patient T cells for reactivity to known antigens and to breast cancer cell line antigens, which will lead downstream to antigen discovery. We have also worked out conditions for direct T cell cloning based on TCR sequence analysis.

15. SUBJECT TERMS

Breast cancer, immunotherapy, vaccine, antigens

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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Table of Contents

INTRODUCTION

The immune response offers exquisite specificity and the potential to target tumor cells without harming normal cells. Inducing an effective immune response via therapeutic vaccines for cancer had been a promising but elusive goal for years. For breast cancer (BC), vaccine efforts have largely focused on eliciting immune responses to HER2. While HER2 is generally assumed to be a good antigen in HER2-overexpressing tumors, HER2-specific T cells exist at very low levels (less than 0.1%) in peripheral blood of such patients (Inokuma, dela Rosa et al. 2007). Hierarchy of the T cell repertoire and negative selection can shape immune responses in ways not readily predictable from protein expression levels alone. Thus, targeting a single antigen such as HER2 in breast cancer is likely to be insufficient - instead we need a repertoire of multiple immunologically validated T cell antigens present in breast cancers that can be deployed in a patient-specific manner. Research has focused on stimulating T cells using many pathways including the T cell antigen receptor (TCR), via co-stimulatory pathways, and manipulating the tumor environment. To optimally activate pre-existing anti-tumor T cells in BC patients, the antigens to which these T cells target must be determined. It is now recognized that invasive ductal carcinoma of the breast is a heterogeneous disease consisting of several major molecularly defined subtypes, including Luminal A, Luminal B, HER2+, and Basal (also known as 'triple-negative', and includes the 'claudin-low' subset). These subtypes have distinct clinical, genomic and proteomic features, and it is becoming clear that there are differences between BC subtype and response to specific therapeutic agent. These results, combined with the differences in gene expression that define the distinct subtypes, make it likely that each BC subtype elicits immune responses via distinct sets of antigens, and may evade T cell-mediated killing by distinct mechanisms. Based on these newly discovered features of BC and the host immune response, this project seeks to develop a robust portfolio of immunologically validated antigens for the major BC subtypes, including those that target breast cancer stem cells, that can be used in a patient-specific manner for therapeutic vaccination, as well as to identify drugs that can synergize with these novel immunotherapies. The ultimate goal is to match these antigens and drugs to each patient's tumor subtype, thereby treating each patient with the most potent combinations and opening the door to personalized immunotherapy for breast cancer. This multi-ream project will use a number of novel immunological approaches to look for evidence of BC subtype specific tumor-reactive T cells within the tumor and/or tumor-draining lymph nodes (TDLNs) including isolating, expanding and cloning tumor-reactive T cells which will culminate in a robust portfolio of immunologically validated antigens for the major breast cancer subtypes, including those that target breast cancer stem cells. We seek to expand and enhance the function of these pre-existing anti-tumor T cells in patients by discovering their natural antigens, and identifying mimotopes that broadly activate them with even higher potency. Furthermore, we will enhance the efficacy of these T cells by identifying existing drugs that promote cancer cell apoptosis but have little or no negative effect on T cells. All of these antigens and agents can be matched to each patient's tumor subtype and other molecular characteristics, thereby opening the door to personalized immunotherapy.

BODY:

Our team is now complete and consists of 1 post-doctoral fellow, 2 research associates, and 1 lab technician. We work closely with our surgery, oncology and pathology colleagues to obtain samples from the operating room to pathology and to my laboratory. This process is done via an honest broker at CoH as we do not obtain protected health information (PHI). Based on advice from our CoH IRB director, we changed our study to Not Human Subject Research (NHSR) in the past year. This modification has been approved by the CoH IRB and is pending approval by the DoD HRPO. In addition, we continue to refine our protocols to maximize recovery of immune cells from tumor and lymph node specimens, and to optimize methods for analysis of fresh samples by flow cytometry. Below is a summary of our progress in relation to our proposed SOW tasks:

Identify immunologically validated antigens by determining antigens recognized by anti-tumor T cells from patients with major subtypes of breast cancer.

1. Generate reagents and identify conditions for experiments to follow: months 1-40, Lee, Slansky, and Spellman
2. Enroll 100 patients with all major breast cancer subtypes from the City of Hope Cancer Center (CoH): months 1-36, Lee
3. Process patient samples (blood, TDLNs, tumor): months 1-38, Lee
4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee
5. Generation and initial analysis of T cell clones: months 1-40, Lee
6. Determine antigens as subtype-specific, stem-specific, or shared (Aim 4a): months 12- 40, Lee, Slansky and Spellman
7. Identify antigens that target breast cancer stem cells (Aim 3b): months 12-40, Lee, Slansky and Spellman

Patient Enrollment and Sample Acquisition
Our progress thus far has focused on tasks 1-5. Patient samples that have been acquired have been used for testing and optimizing conditions. As of August 2013, we have enrolled 11 breast cancer patients into this study prior to obtaining Not Human Subjects Research (NHSR) determination through our IRB. Obtaining blood is essential to this study to generate source cells for both monocyte-derived dendritic cells and EBV transformed B cells, which will be used as autologous antigen presenting cells (APCs) to present tumor antigens to T cells. Patient characteristics are summarized in Table 1. NHSR determination involves an honest broker that further decodes specimens given to us (i.e., BC1, BC2, etc.) from the original RPN code, thereby removing possible access for us to PHI. The honest broker holds the key to the master coded list and is not involved in our research processes. We are in the process of updating our IRB application and protocol where we describe that we will also strip the previously enrolled research participant numbers from our enrolled cohort and from our records.

All participants were without a history of any immune disorder prior to breast cancer diagnosis and had their surgical treatments at City of Hope (CoH). Written informed consent had been obtained from all participants according to CoH and HIPAA regulations through a tissue banking protocol. Patient peripheral blood samples, breast tumor tissue, tumor draining lymph node (TDLN: non-sentinel lymph node and/or sentinel lymph node) were collected and have been utilized for research purposes.

The Slansky/Kappler and Lee teams have recently re-designed a flow chart for the distribution of patient cells between labs (Figures 1-3). The flow charts represent an ideal situation, which are heavily dependent on the sample sizes we receive and success of upstream protocols.

Process of Patient Samples

HLA typing
We have arranged to have patients' peripheral blood mononuclear cells (PBMCs) DNA HLA typed through the Histocompatiblity lab here at CoH. CoH's Histocompatibility Laboratory is fully accredited by The American Society of Histocompatibility and Immunogenetics (ASHI), College of American Pathologists (CAP), and Clinical Laboratory Improvement Amendments (CLIA 88). They will carry out the typing using the sequence-specific oligonucleotide probe (SSOP) method. The SSOP method allows the HLA lab to define the HLA type of our patient subjects to the allele level (so called '4 digits'). Initially we are requesting for the allele level typing of subjects for only HLA-A2 and HLA-DR, but information on other alleles is available at a later date if desired. Thus far, we have sent 5 samples to be HLA typed at CoH and have sent 6 samples to the Slansky/Kappler group for TCR sequencing. HLA samples sent for typing are summarized in Table 2.

Identify and isolate anti-tumor T cells from TDLNs and tumor samples

Cell Isolation
In our previous progress report, we noted that using Dynall Flow Comp CD3 bead based isolation kits achieved acceptable purity, but Dynall Flow Comp CD4 and CD8 kits needed further optimizing to achieve acceptable recovery. After various attempts to do so, we decided switching to Stem Cell Technologies as our supplier of bead based isolation kits would be the best course of action. Currently we are more than satisfied with the efficiency of their kits, including CD3$^+$ isolation, CD4$^+$ isolation, and CD8$^+$ isolation (Figure 4).

While T cell isolation from tumor cell populations works effectively to attain a highly pure population of T cells within the lymphocyte population, tumor cells and tumor debris may remain. Such purity is sufficient for T cell sequencing or even analysis by flow cytometry, but makes single cell plating for T cell cloning purposes difficult. Since a new direction we are pursuing is the direct cloning of CD8$^+$ T cell clones from tumor tissue, we investigated ways to 'clean up' our starting CD8$^+$ T cell population. Lymphocyte cell purity was examined by flow cytometry either immediately or after an overnight incubation of the cells on a tissue culture plate, with the hope that tumor cells and tumor debris would attach to the plastic plate (Figure 5). We observed that the overnight culture significantly decreased the amount of tumor cells and cell debris present in the cell suspension and also significantly increased the purity of T cells isolated following the incubation (data not shown). We also investigated the potential use of protocols developed by other groups (Dudley, Wunderlich et al. 2003), involving either leaving undigested tumor chunks in high dose IL-2 (6000 IU/ml) and allowing the tumor T cells to 'crawl out' or placing digested tumor cell suspensions in high dose IL-2. While these protocols were extremely successful in generating large number of tumor T cells, we suspect that the T cells may be proliferating in addition to crawling out. Because of these suspicions, we decided that a combination of plating isolated CD8$^+$ T cells overnight on plastic with FACS single cell sorting of CD8$^+$ T cells into wells would be the most optimal approach going forward. While limiting dilution cloning seems to work effectively, the guarantee offered by FACS sorting that there will be one cell per well is more than what limiting dilution can offer.

Generation of Antigen Presenting Cells (APCs)
The generation of antigen presenting cells (APCs) is a critical component of this project. As mentioned above, we have identified two possible sources of APCs: Epstein Barr Virus (EBV) transformed B cells and monocyte-derived dendritic cells (mDCs), both of which will be generated from patient autologous PBMCs. EBV transformed B cells become an immortal cell line capable of relatively easy expansion and successful cryopreservation, thus being a plentiful source of autologous APCs. However, the transformation process is slow, taking at least 6 weeks, and EBV transformed B cells lack the degree of co-stimulation molecules that DCs have. DCs are potent professional APCs. However, generation of mDCs is dependent on the starting number of monocytes, and they generally do not expand in culture and are less resilient to cryopreservation. Due to the benefits and drawbacks of each APC type, we have decided to attempt to generate both for each patient enrolled into this study. mDCs will be the preferred APC for presentation of tumor antigen, but EBV transformed B cells will be available if the mDC approach is unsuccessful.

Our early attempts to generate EBV transformed B cells from healthy controls resulted in less successful transformation (25% success rate) than what we would prefer (95% success rate). Because of this, we made a new stock of EBV containing supernatant by expanding the B95-8 EBV generating cell line for several weeks followed by lysis using a freeze-thaw method. We expect that this new stock of B95-8 supernatant has a higher titer of EBV, and is therefore more potent in transforming B cells, as we expanded the cells for a longer period of time and at a higher concentration. Additionally, we have modified our protocol to include FK-506 in addition to Cyclosporin A, both of which are similar immunosuppressive drugs that interfere with proper T cell function. Using this modified method and new EBV containing supernatant, our current success rate for transformation is between 60% and 80%.

Since detection of successful B cell EBV transformation cannot be easily determined until 5 to 6 weeks after beginning the co-culture of EBV supernatant and target B cells, we have incorporated a flow cytometry based method for detecting successful transformation early on. It has been demonstrated that upon successful transformation, CD23 (FcεRII) and CD58 (lymphocyte function-associated antigen 3) are upregulated on EBV transforming cells 3 days post transformation (Megyola, Ye et al. 2011) (Figure 6). Although we have improved the successful transformation rate, we still hope to achieve closer to 100% successful transformation through continued optimization. Since our current protocol uses total PBMCs as a source of target B cells, we plan on using purified B cells to increase the target cell numbers. Furthermore, we have acquired a stock of EBV containing supernatant from ATCC, which has a defined EBV titer level. This positive control should allow us to confirm the success rate of our transformation and the quality of our own EBV containing supernatant.

As of yet, we have not tested EBV transformed B cells for their APC function, though this is our plan in the coming year. We have been able to generate HLA-A2+ EBV transformed B cells, which can be used for testing as APCs in the context of our HLA-A2+ restricted T clones as described below. It is likely that using these B cells as APCs will require either electroporation, osmotic shock or some other method to allow antigen to be taken up into the endogenous antigen pathway for presentation on class I molecules to CD8$^+$ T cells.

To generate mDCs, isolated monocytes are cultured with Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (O'Neill and Bhardwaj 2005). After 5-6 days of culture, immature DCs can be matured using a variety of methods. We compared two methods for maturing mDCs: 100ng/ml lipopolysaccharide (LPS) versus monocyte maturation cocktail (MMC) composed of 5ng/ml IL-1β, 150ng/ml IL-6, 5ng/ml TNF-α and 1ug/ml prostaglandin E2 (PGE2). After 24 or 48 hours we assessed DC maturation markers CD83, CD86, and MHC Class I and II molecules (Figure 7). Both LPS and the MMC yielded DCs with elevated expression of all maturation markers. Compared to LPS, MMC yielded higher CD86 and CD83 expression and lower expression levels of MHC molecules. Since the majority of DC literature uses some version of the MMC to mature DCs, we adopted it as our standard DC maturation method. However, once our protocol for identification of tumor reactive T cells is fully optimized, we may re-compare LPS maturation to MMC maturation on a functional level.

Once mDCs are generated they will be used as APCs for tumor antigen to patient T cells. However, since classical mDC generation takes 5-7 days and it is less than ideal to culture T cells for that long, we investigated alternative methods of mDC generation. The 48 hour 'Fast mDC' protocol involves the same DC differentiation cytokines, but an early endpoint for differentiation (Dauer, Obermaier et al. 2003) . We conducted a pilot study where we differentiated mDCs for 48 hours, matured them overnight with MMC, and assayed various maturation markers. Compared to the classical DC differentiation protocol, Fast DCs have lower expression of activation markers (data not shown), although still up-regulated. We therefore concluded that this system can be utilized if necessary.

T cell culture conditions
As described in our previous report, prior to screening patient lymph node (LN) and tumor T cells for antigen reactivity we expanded them for 6-7 days using CD3/CD28 expansion beads to increase cell numbers. These T cells could then be co-cultured with autologous mDCs previously pulsed with tumor cell line lysates, which were generated by the Spellman group. After co-culture the T cells were examined by flow cytometry for CD107 mobilization and the upregulation of activation markers CD69, CD137, and CD154 (CD40L) in order to identify T cells that are reactive to the tumor lysate. CD107 mobilization reflects degranulation by CD8$^+$ T cells, while CD154 is a CD4$^+$ activation marker. CD69 and CD137 are activation markers on both CD4$^+$ and CD8$^+$ T cells that are early and late activation markers, respectively.

Upon analysis of these markers by FACS we observed high background levels of CD107 mobilization on CD8+ T cells and CD154 expression on CD4$^+$ T cells in our sample that was not stimulated with antigen (Figure 8A). To investigate if CD3/CD28 bead based expansion was inducing the high background, we conducted a pilot

study where we isolated T cells from 3 healthy donors' PBMCs and cultured them for 5 hours or 3 days under the following conditions: a) media alone, b) in the presence of 100IU/ml IL-2, c) in the presence of 12.5pg/ml IL-7 or, d) with CD3/CD28 expansion beads (1:1). Interestingly, we observed increased CD107 mobilization in CD8$^+$ T cells and increased CD154 on CD4$^+$ T cells after 5 hours in the presence of CD3/CD28 expansion beads compared to media alone (Figure 9). Furthermore, CD107 mobilization significantly increased after 3 days in the presence of CD3/CD28 expansion beads on CD 8$^+$ T cells as well as increased expression of CD154 and CD137 on CD4$^+$ T cells (Figure 10), producing false-positive results for tumor reactivity. IL-2 and IL-7 presence, however, has little to no effect on CD107, CD154, and CD137 expression levels. Since the effect of CD3/CD28 beads on CD107 mobilization and CD154 expression levels was observed to last for several days even after removing the T cells from the beads (data not shown), we have decided to simply culture freshly isolated T cells in IL-2 alone until mDCs are ready for use as APCs (5-7 days). We have also verified that these culture conditions result in high viability and recovery of the T cells (data not shown).

Tools/ Systems
Our initial screening of patient sample T cells for reactivity to breast cancer cell line antigen presented by DCs did not yield significant numbers of T cells that appeared positive for various activation markers. To optimize these protocols and verify that they worked properly in our system for identification of T cell reactive clones, we are using T cell clones previously generated in our lab. These clones are CD8+ T cells specific for either the MART1 (specifically the M27 peptide) or gp100 (specifically the g209 peptide) proteins expressed by cancer cells. In conjunction with this we have a tumor cell line, which expresses both these proteins and our effectively killed by the T cell clones (Figure 11C). Furthermore, artificial APC cell lines or HLA matched DCs elicit an effective degranulation response from the T cell clones when pulsed with target peptide (Figure 11B). To mimic our planned experimental approach for our breast cancer patient samples, we made a lysate of the cancer cell line using a freeze thaw method. From 68 million cells we obtained 6.8mg of protein, as measured by a Bradford assay. Pelleting the insoluble portion of the lysate resulted in a soluble portion with a concentration of 5.8mg/ml, as compared to the total lysate concentration of 9.66mg/ml. While most of the literature describes using only soluble lysate as antigen, we were unsure which portion of the lysate our unknown antigens of interest would be in. Because of this we are currently testing both types of lysate as antigen.

T cell Antigen Screening
Methods for the identification of tumor reactive T cells have been our focus over the last year. To do so we have identified two potential pathways for screening patient T cells against breast cancer antigen: breast cancer cell line lysate and electroporation of breast cancer RNA. Both pathways make use of autologous DCs derived from PBMC monocytes and breast cancer cell lines that the Spellman lab possesses.

Our primary approach for introducing antigen into DCs is to generate mature mDCs pulsed with breast cancer cell line derived lysate. These DCs are then mixed with patient T cells to screen for tumor reactivity. Using the lysate generated from the cancer cell line and our cancer specific T cell clones, we have tested various conditions to optimize this pathway. Various conditions tested include adding the cancer cell line lysate at the same time as the MMC or adding the MMC 24 hours after adding the cancer cell line lysate, using the soluble portion of the lysate or using total lysate, and several DC: T cell ratios. We have also compared several time points and activation markers beyond a 4 hour CD107 mobilization assay, such as CD107 mobilization after 12 hours, CD69 expression after 6 hours, and CD137 expression after 24 hours. However, we have not found the proper conditions for stimulation of the cancer specific T cell clones (Figure 12). We do know that they respond well to both their target peptide and the cancer cell line itself. Continued work on this protocol may involve using LPS and IFN-γ maturation of DCs which may yield more cross-presentation, quantifying the level of MART1 and GP100 in our cancer cell line lysate using a western blot, and/or purchasing purified MART 1 and GP100 protein to test if the DCs are effectively cross-presenting antigen. Furthermore, we have plans to generate Her2+ specific T cell clones so that we can test these conditions using the cancer cell lines from the Spellman lab.

Electroporation

Due to our limited success thus far with using cell line lysate as antigen source, we are currently optimizing the introduction of tumor antigens into APCs by electroporation. Electroporation is a transfection method in which an electrical current is pulsed across the cell, which perturbs the plasma cell membrane creating temporary pores. This increases the electrical potential across the cell which allows charged molecules like protein/RNA/DNA to be driven across the membrane. The Life Technologies Neon Transfection electroporation system is available in a CoH shared facility. This system has recommended settings that have been optimized for a human dendritic cell electroporation protocol. Additionally, we have purchased enhanced green fluorescent protein (eGFP) mRNA to be used as a positive control to assess the efficiency of electroporation.

mDCs were generated as described above and then electroporated with 10ug of eGFP mRNA. 24 hours post electoporation, we assessed the recovery and viability of cells, as well as the efficiency of the electroporation by analysis of eGFP expression using flow cytometry. We were able to achieve high cell recovery, high viability (data not shown) and a 97.8% efficiency of transfection (Figure 13). Various reports in the literature use total RNA, mRNA, or cell lysate for the electroporation of antigen into DCs. Since we now have a successful protocol for expressing foreign protein in mDCs, we plan on testing this protocol using a system of mDCs, our cancer specific T cell clones, and either total RNA, mRNA, or cell lysates isolated from a cancer cell line.

Generation and initial analysis of T cell clones

Direct T cell Cloning
As discussed above, we have had difficulty with identifying tumor antigen reactive T cell clones from fresh cells. While we continue to optimize these assays, we looked for other ways to continue to elucidate the antigen specificity of tumor T cell populations. Previous sequencing results in our lab showed that the top 10 most dominant T cell clones in tumor samples tend to have a frequency of over 1% (Figure 14). With this information in mind, a statistician here at CoH analyzed the number of T cell clones necessary to generate in order to successfully obtain all clones with a frequency of at least 1%. As Table 3 shows, by generating between 200 and 300 clones there is a probability between 87% and 95% that clones with a frequency of 1% and above in the starting population will be acquired. We therefore plan to directly single cell sort and clone CD8$^+$ T cells from tumor. However since not every T cell clone will proliferate equally or perhaps not at all, we will also need to compare the TCR sequences of the generated clones to the high throughput sequence data obtained by sequencing the total T cell repertoire. Therefore with each clone generated, we can cross-reference the frequency of the starting T cell repertoire. The T cell clones can then be screened against known antigens or against unknown antigens using the Slansky/Kappler lab bacteriophage peptide library.

Recently we have participated in several days of training for approval as 'self-sorters' at the Analytical Cytometry Core here at the City of Hope. This status allows us more flexibility for sorting, which is desirable to the unpredictability of obtaining tissue samples in the lab.

Summary of Plans for Future Work

Project team members have recently returned from a productive meeting with our collaborators at the National Jewish Health Campus. Additionally a CoH postdoctoral fellow took the opportunity to do some training at the Slansky lab for a day to learn various aspects of creating tetramers, transfected cell lines, and other molecular methods. Furthermore, the trip allowed for excellent extended discussion of experimental approaches and strategies.

As such, we have outlined a schematic for the experimental flow of our patient tissue cells (Figures 1-3). The current experimental setup is an evolution of our original strategies that involves new assays for screening patient T cells for specificity to known antigens and more stress on the sequencing of TCRs to examine the T cell repertoire.

Patient LN and tumor T cells will be screened against various known antigens, such as HER2, hTERT, CEACAM5, and NY-ESO-1. To do so we will screen CD8$^+$ T cells for reactivity, using a CD107 mobilization assay as a readout, against either artificial APC cell lines transfected to express these antigens, which have been created by the Slansky team, or against artificial APC cell lines pulsed with immunodominant peptides of the same antigens. Similarly we are working on creating a tetramer array to screen patient T cells in a high throughput way for specificity to various known antigens.

These cell based assays that the CoH team will lead will complement well with the TCR sequencing work that the Slansky/Kappler team will lead. Identification of the complete T cell repertoire of patient blood, tumor, and TDLN tissues will be created using either a single cell sequencing (scSeq) approach or by high throughput sequencing of TCR beta and alpha chains separately, both of which are currently being led by the Slansky/Kappler teams. This T cell repertoire analysis information will be cross referenced with the TCR sequence data of tumor antigen reactive or specific T cells. Upon completion we will have obtained both the TCR sequences for known antigens and the TCR sequences for unknown antigens, which can then be used to screen against the bacteriophage peptide library the Slansky/Kappler team has created to identify those unknown antigens.

Currently we are focused on HLA-A2+ patients due to the HLA-A2 restriction of the various TCR screening tools generated by the Slansky/Kappler team. Thus, in our work so far we have primarily focused on CD8$^+$ T cells for similar reasons. We do, however, plan on completing some limited screening of CD4$^+$ T cells and to isolate and expand clones of CD4$^+$ T cells that react to breast cancer cell line antigens in the future.

Outline of the project plan for the next 12 months

- Continue to finalize optimal EBV transformation protocol from PBMCs
  - Determine if EBV transformed B cells properly present antigen to CD8$^+$ cells through electroporation or osmotic shock protocols
- Continue to optimize antigen presentation by mDCs for the identification of tumor reactive T cells
  - Determine if electroporation or pulsing of cell line lysate antigen are effective protocols.
- Create expanded T cell clones of the most frequent tumor T cell clones
  - Screen, along with the Slansky/Kappler team, for antigen specificity
- Screen patient LN and tumor T cells for antigen specificity to known antigens
- Continue providing T cell samples to the Slansky/Kappler team for sequencing of the TCR repertoire
- Continue providing tumor tissue samples to the Spellman group for RNA sequencing in the hopes of identifying potential T cell target epitopes

Personnel
1. Peter P. Lee, MD – project PI (40% effort)
2. John Yim, MD – CoH Surgical Oncology (5% effort)
3. Joanne Mortimer, MD – CoH Medical Oncology (no salary requested)
4. Jing Zhai, MD, PhD – CoH Pathology (no salary requested)
5. Colt Egelston, PhD – post doc (100% effort)
6. Diana Simons – Research Associate II (95% effort)
7. Emily Andersen – Research Associate I (50% effort)
8. Grace Jimenez – Lab Technician (100% effort)

KEY RESEARCH ACCOMPLISHMENTS

- Further optimized cell isolation protocols from patient PBMCs, TDLN, and tumor
- Optimized protocols for generation of monocyte-derived dendritic cells
- Continued optimization of EBV Transformation of B cells for use as a long-term autologous APC source
- Optimized conditions for successful short term culture of TDLN and tumor T cells
- Resolved issue of high background levels of activation markers on T cells caused by CD3/CD28 expansion beads
- Optimized flow cytometry based identification of antigen-reactive T cells
- Optimized single cell cloning of $CD8^+$ and $CD4^+$ T cells
- Optimized protocol for electroporation based transfection of dendritic cells
- Established antigen specific systems for studying T cell activation by APCs
- Identified a strategy to culture and expand the most frequent clones from tumor T cells
- Established, along with the Slansky/Kappler team, a strategy for screening patient T cells against known tumor antigens

REPORTABLE OUTCOMES

None at this time, but the teams are preparing our first joint manuscript for submission soon.

CONCLUSION:

Over the last 12 months of this award, we have focused on the optimization of various systems for the identification of tumor-reactive T cells. Our team has tested and optimized protocols for the isolation of several immune cell types, the generation of antigen-presenting cells, and the culturing of T cells. We have also optimized protocols and systems to screen T cells for reactivity to cancer cell line antigens and the cloning of T cells. We have began screening patient T cells for reactivity to known antigens and to breast cancer cell line antigens, which will lead downstream to antigen discovery. We have also worked out conditions for direct T cell cloning based on TCR sequence analysis.

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APPENDICES:
None at this time

SUPPORTING DATA:

Table 1. Patient characteristics

Code	Prior Therapy	Diagnosis	ER/PR/Her2	Molecular Subtype	Stage
BC# 1	N	IDC	+/+/-	Lum A	IIa
BC# 2	N	ILC	+/+/-	Lum A	IIa
BC# 3	N	IDC	+/+/-	Lum A	I
BC# 4	N	IDC	+/+/-	Lum A	I
BC# 5	Y	IDC	+/+/-	Lum A	IIIc
BC# 6	N	IDC	+/+/-	Lum A	IIa
BC# 7	N	IDC	+/+/-	Lum A	IIb
BC# 8	N	IDC	+/+/-	Lum A	IIa
BC# 9	N	IDC	+/+/-	Lum A	Unknown
BC# 10	N	ILC	+/+/-	Lum A	Unknown
BC# 11	N	IDC	+/+/-	Lum A	Unknown

Table 2. Samples sent for HLA typing		
Sample Label	HLA-A Type	HLA-DRB1 Type
A	02:03	04:05
B	01:01	04:02
C	02:01	12:02
D	02:01	09:01
E	02:01	01:01
F*		

* Not sent for typing due to CD8^{+} T cell purity

Table 3. Detection probability of clones based on their frequency. A CoH statistician created this table which identifies the detection rate given the frequency of a given clone if a given number, n, of clones are generated.

$$
\begin{aligned}
f &= \text{Relative frequency of TCR sequence} \\
n &= \text{number of sequences sampled} \\
q &= (1-f)^n, \text{ probability of missing the TCR in a sample of } n \\
1-q &= \text{"Detection" probability (single sample)} \\
(1-q)^2 &= \text{"Reliable," detection probability} \\
q(1-q) &= \text{"Unreliable" detection probability}
\end{aligned}
$$

|	f	n	Detection	Reliable	Unreliable
1	0.020	100	0.867	0.752	0.115
2	0.010	100	0.634	0.402	0.232
3	0.005	100	0.394	0.155	0.239
4	0.002	100	0.221	0.049	0.172
5	0.001	100	0.095	0.009	0.086
6	0.020	200	0.982	0.965	0.017
7	0.010	200	0.866	0.750	0.116
8	0.005	200	0.633	0.401	0.232
9	0.002	200	0.394	0.155	0.239
10	0.001	200	0.181	0.033	0.148
11	0.020	300	0.998	0.995	0.002
12	0.010	300	0.951	0.904	0.047
13	0.005	300	0.778	0.605	0.173
14	0.002	300	0.528	0.279	0.249
15	0.001	300	0.259	0.067	0.192
16	0.020	500	1.000	1.000	0.000
17	0.010	500	0.993	0.987	0.007
18	0.005	500	0.918	0.844	0.075
19	0.002	500	0.714	0.510	0.204
20	0.001	500	0.394	0.155	0.239

Jeff Longmate, COH
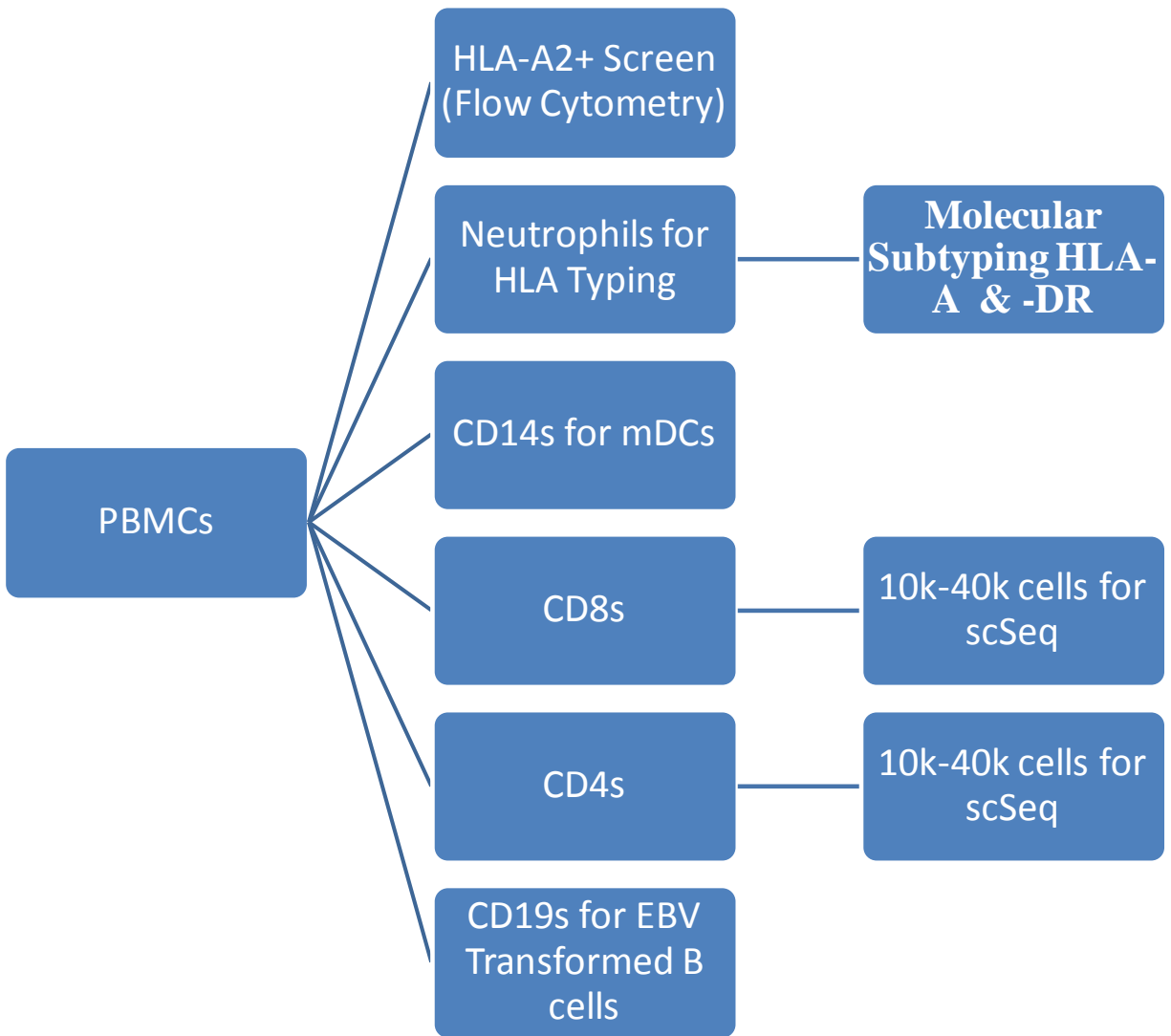
Figure 1. Flow for PBMCs isolated from whole blood for various projects
scSeq denotes single cell sequencing to be done by the Slansky/Kappler group.
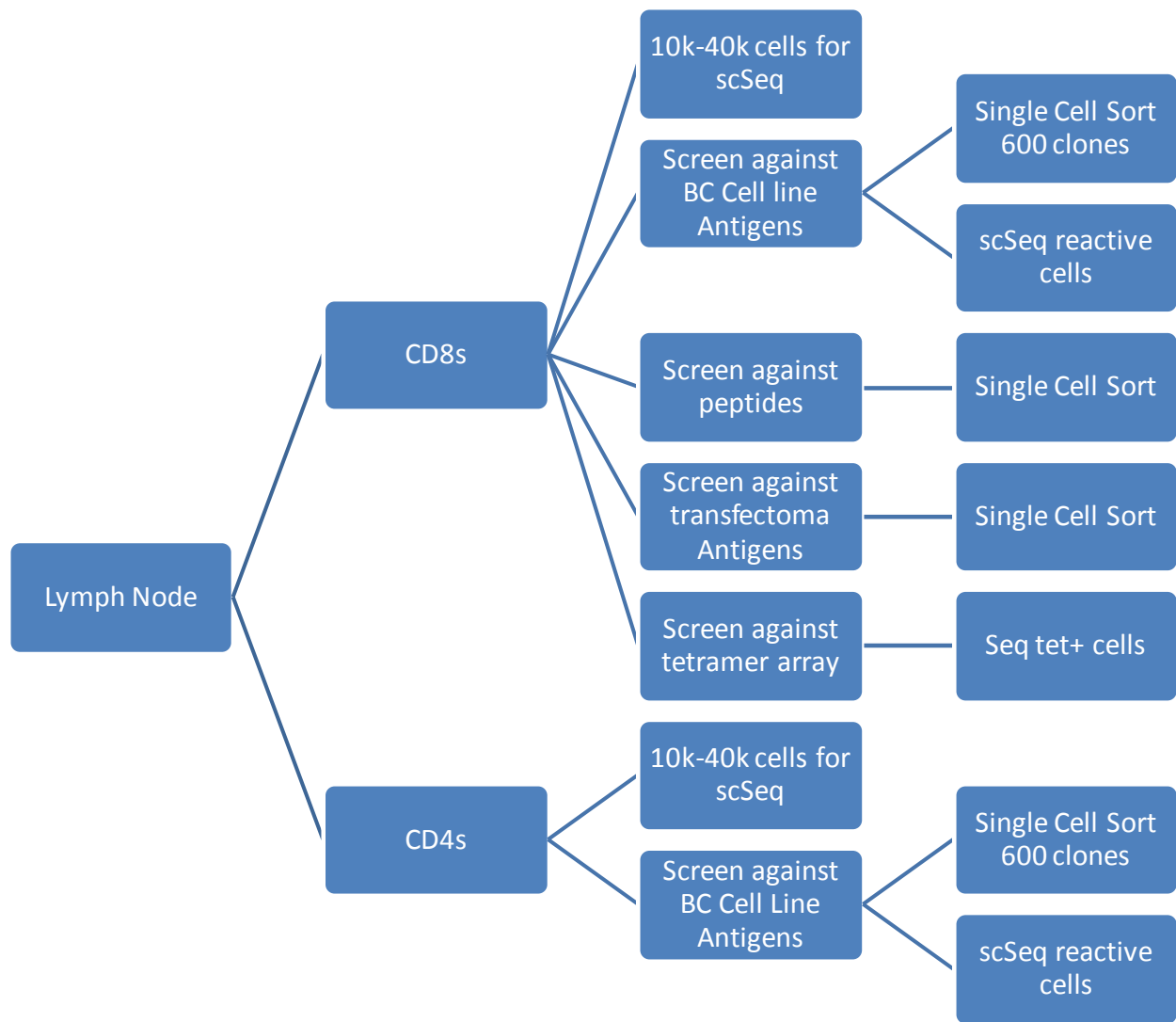
Figure 2. Flow for T cells isolated from lymph node for various projects
scSeq denotes single cell sequencing to be done by the Slansky/Kappler group.
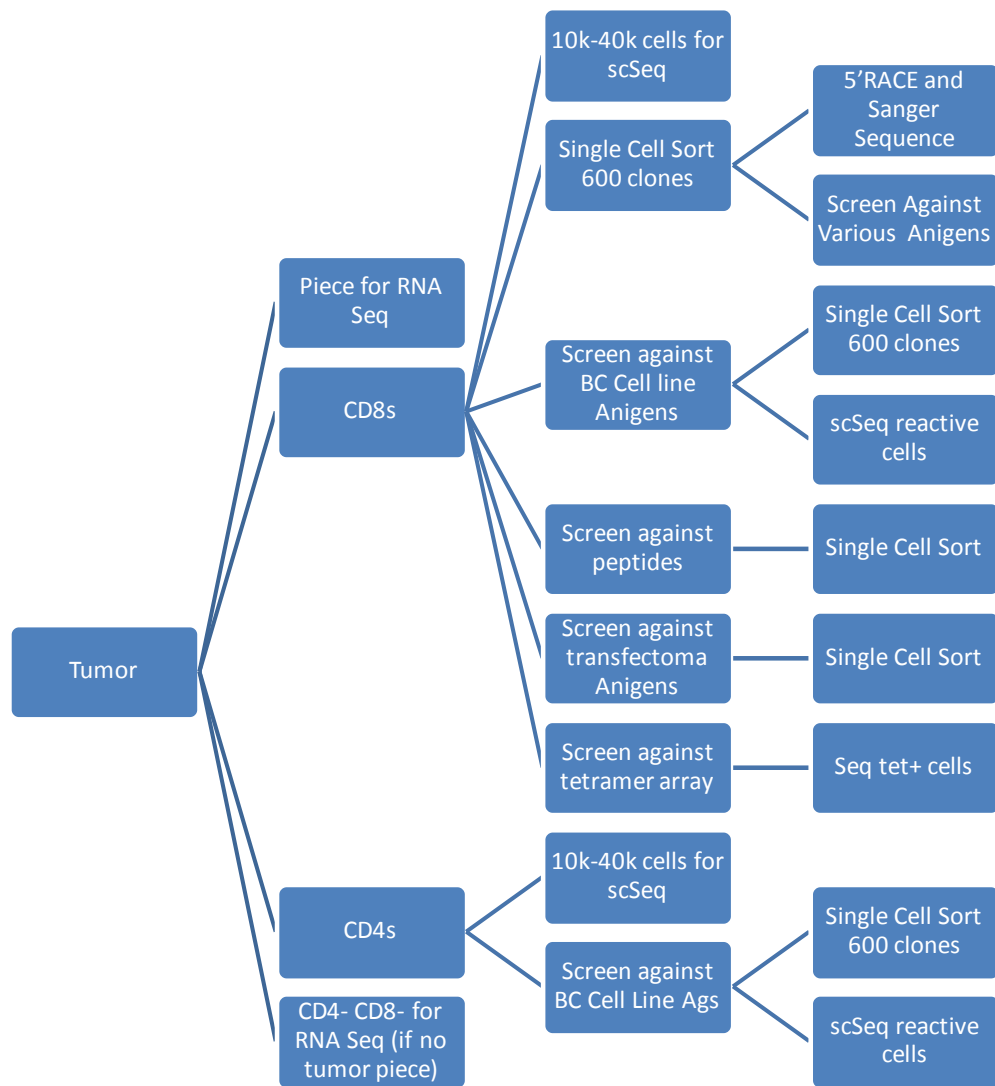
Figure 3. Flow for T cells isolated from tumor for various projects
scSeq denotes single cell sequencing to be done by the Slansky/Kappler group.
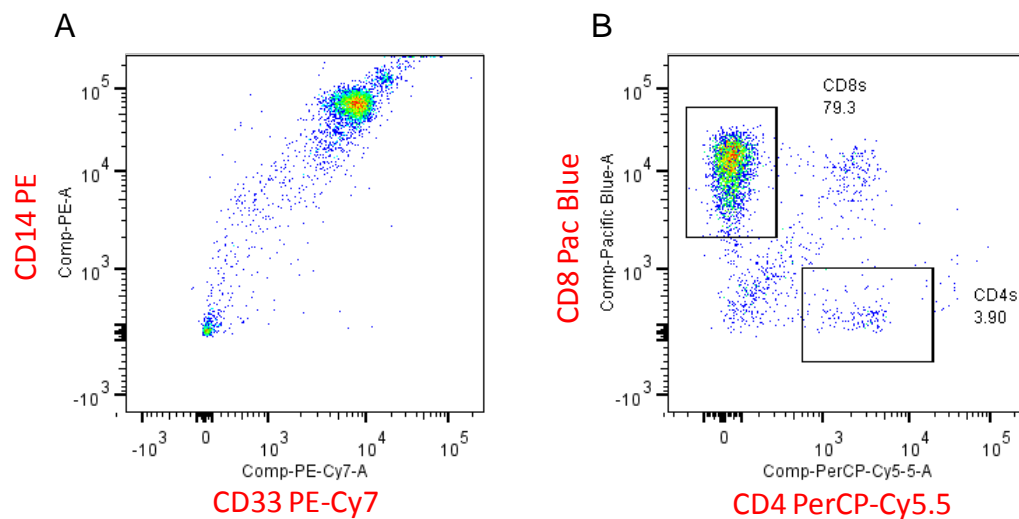
Figure 4. Cell isolation from PBMCs using antibody based kits from Stem Cell Technologies
We routinely perform isolations such as monocytes (A) or CD8$^+$ T cells (B). Cell isolation purity was analyzed by flow cytometry.
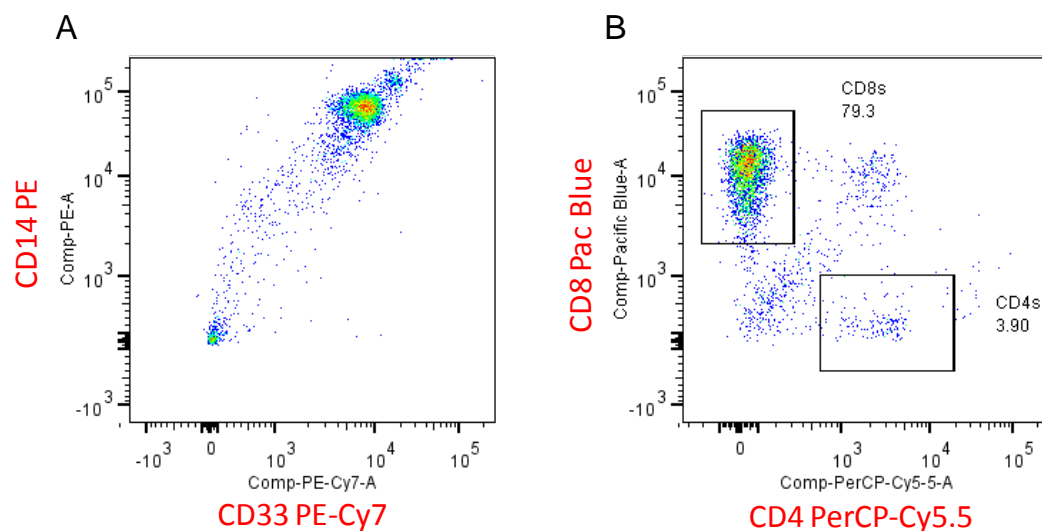
Figure 5. Removal of cell debris and tumor cells from tumor tissue cell suspensions
Tumor tissue was process with collagenase and assessed by flow cytometry either immediately (A) or after an incubation of 24 hours on tissue culture plastic (B).
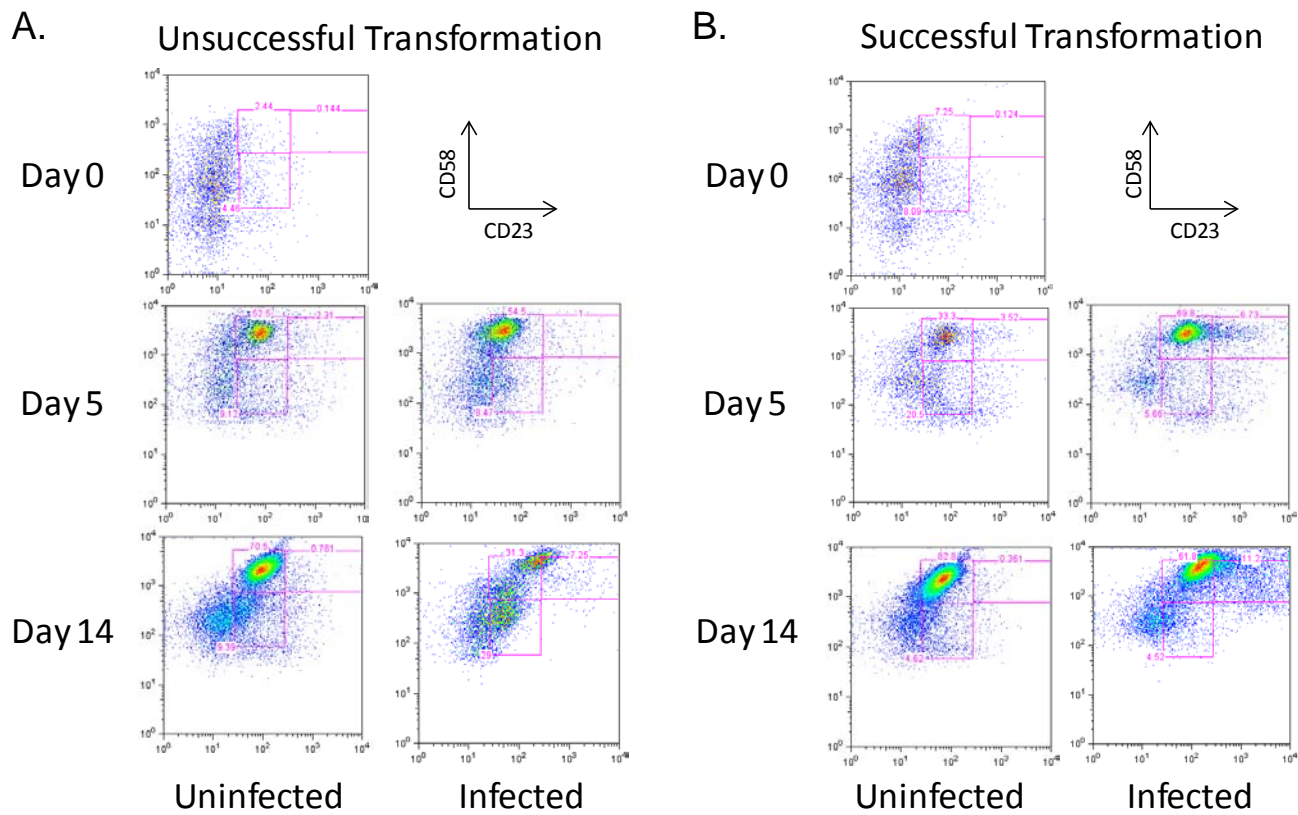
Figure 6. EBV transformation of healthy donors' PBMCs
Five healthy donors' PBMCs were transformed using EBV titer with immune cell inhibitors, FK-506 and Cyclosporin A. Surface markers CD23 and CD58 were evaluated prior to transformation (Day 0), and Day's 5 and 10 post transformation (Infected). Untransformed PBMCs from the same donors were used as negative controls (Uninfected). (A) Representative donor who's B cells unsuccessfully transformed and, (B) Representative donor whose B cells successfully underwent transformation.
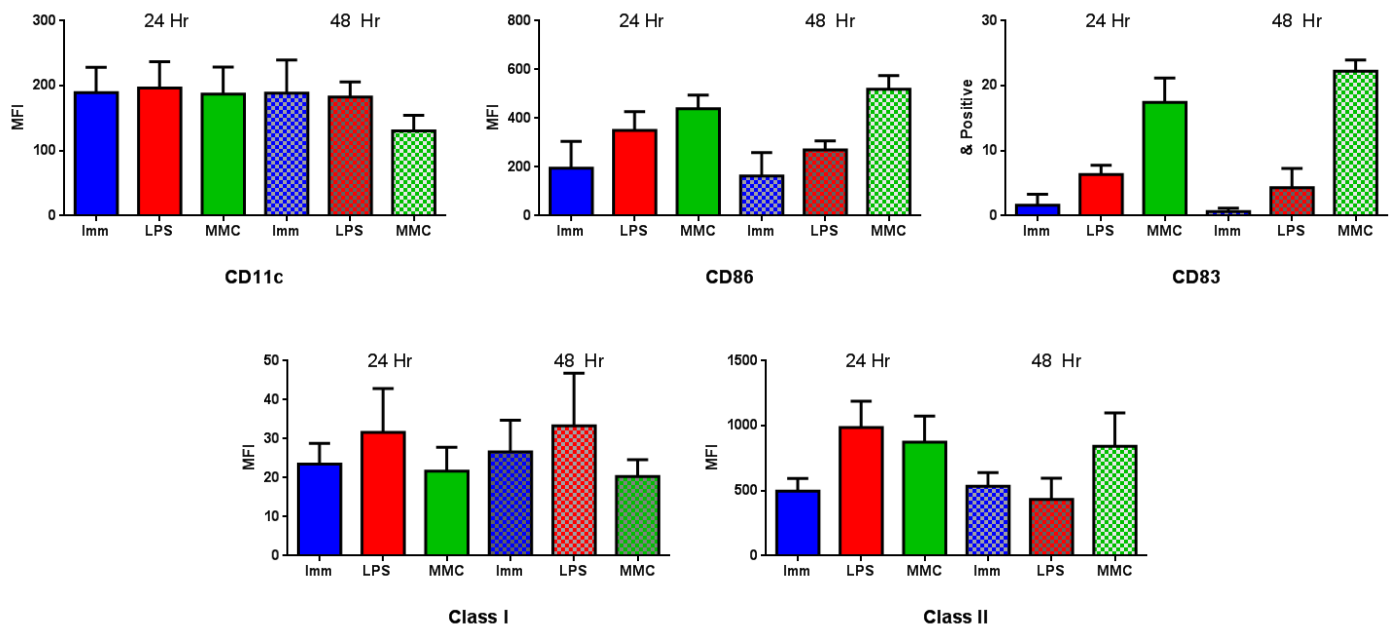
Figure 7. Maturation methods of monocyte-derived dendritic cells
We compared two methods for maturing mDCs. Immature mDCs were matured with 100ng/ml LPS or matured with a monocyte maturation cocktail composed of 5ng/ml IL-1β, 150ng/ml IL-6, 5ng/ml TNF-α and 1ug/ml prostaglandin E2 (PGE2). We compared maturation markers CD11c, CD86, and CD83, as well as MHC Class I and II antigen-presenting molecules after maturing for 24 hours or 48 hours.
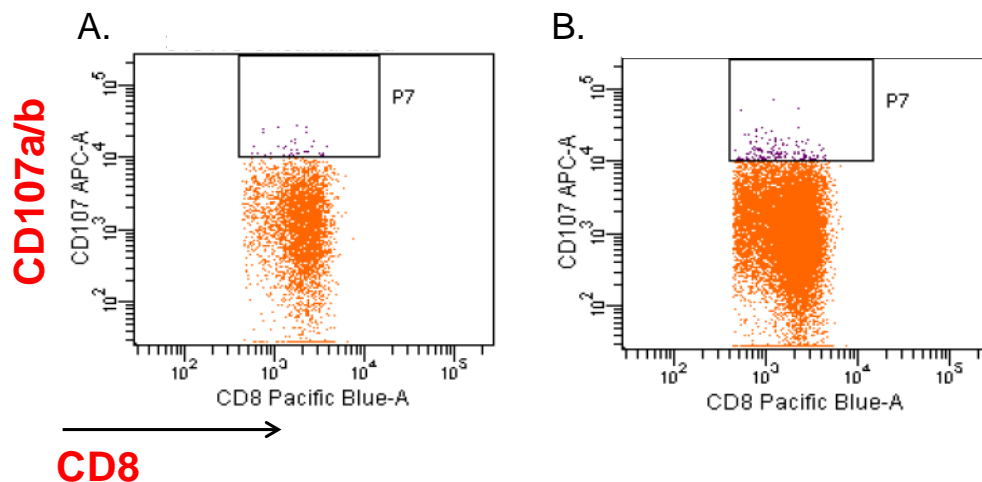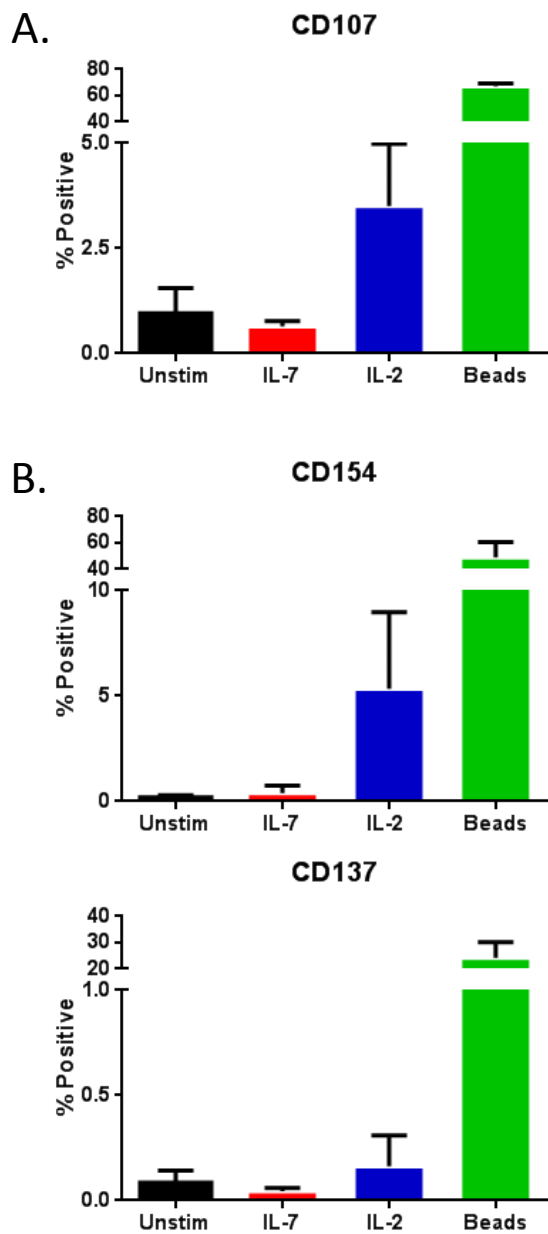

Figure 8. Effects of anti-CD3/CD28 expansion beads on CD107 mobilization in CD8^{+} T cells
T cells were expanded with anti-CD3/CD28 expansion beads (1:1) for 6 days. T cells were harvested and co-incubated with, (A) mDCs not pulsed with tumor lysate (negative control) or, (B) mDCs pulsed with 50ug/ml of tumor cell line lysate, in the presence of CD107 antibody and monensin for 5 hours, followed by surface staining with CD8.
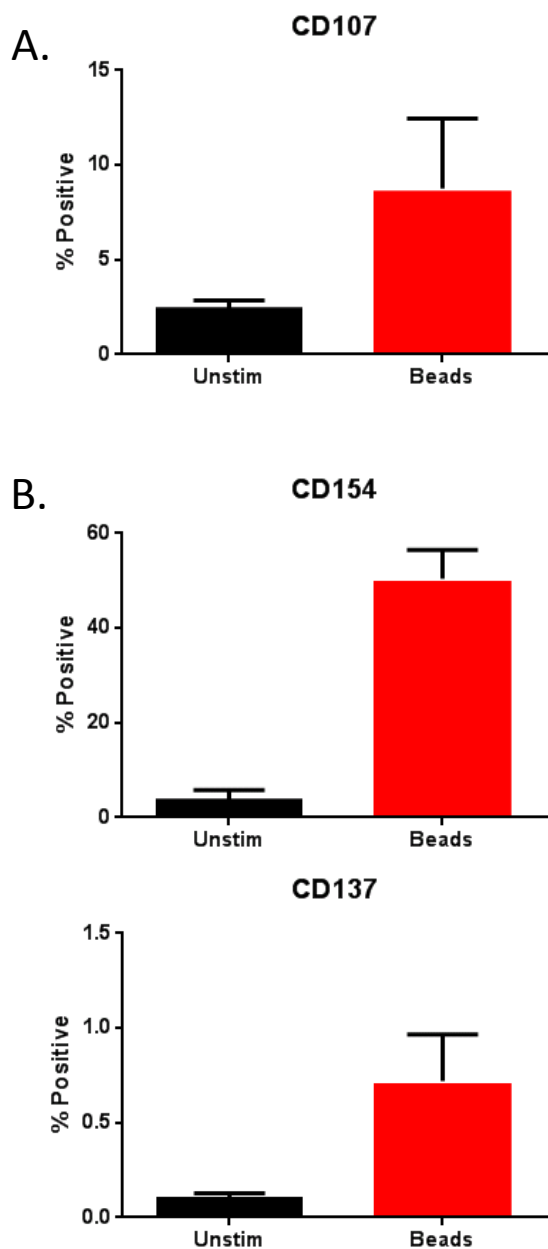
Figure 9. Effects of anti-CD3/CD28 expansion beads in isolated T cells
Isolated CD3^{+} T cells were unstimulated or stimulated with anti-CD3/CD28 expansion beads (1:1) for 4 hours in the presence of CD107 and CD154 antibodies with monensin. After 4 hours, cells were washed and stained with CD137. Cells were assessed for (A) CD107 mobilization on CD8^{+} T cells and, (B) CD154 and CD137 activation on CD4^{+} T cells.

Figure 10. Effects of cytokines and anti-CD3/CD28 expansion beads on isolated T cells
Isolated CD3^{+} T cells remained unstimulated or cultured in the presence of 12.5pg/ml IL-7, 100IU/ml IL-2, or anti-CD3/CD28 expansion beads (1:1) for 72 hours. Cells were washed, stained with CD107 and CD154 in the presence of monensin for 4 hours followed by CD137 staining. Cells were assessed for (A) CD107 mobilization on CD8^{+} T cells and, (B) CD154 and CD137 activation on CD4^{+} T cells.
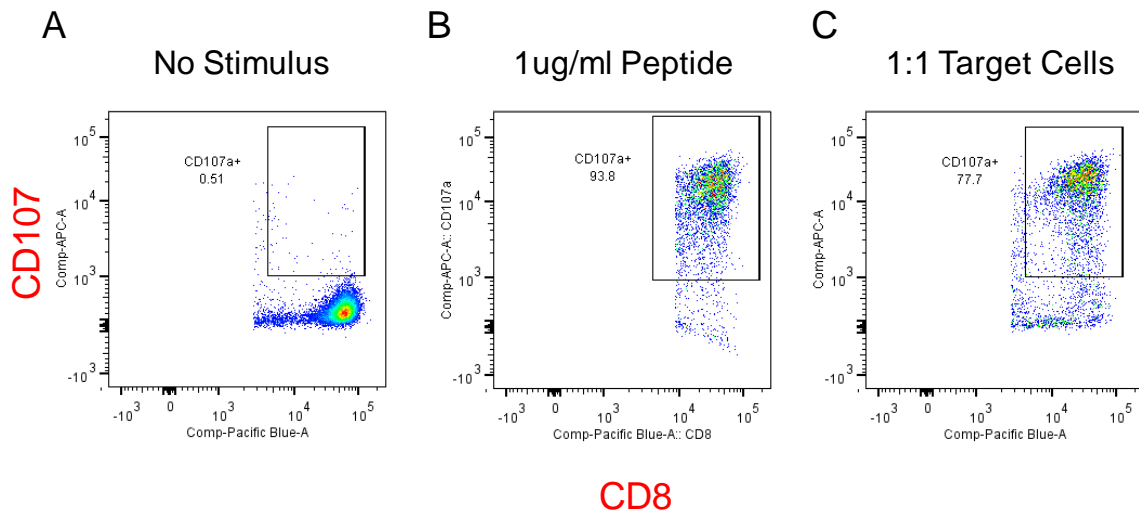
Figure 11. Cancer specific T cell clones are antigen specific
Cancer specific CD8^{+} T cell clones were either left unstimulated (A) or stimulated with an artificial APC cell line pulsed with target peptide (B) or mixed with target cancer cells (C). A 4 hour CD107 mobilization assay was performed and analyzed by flow cytometry.
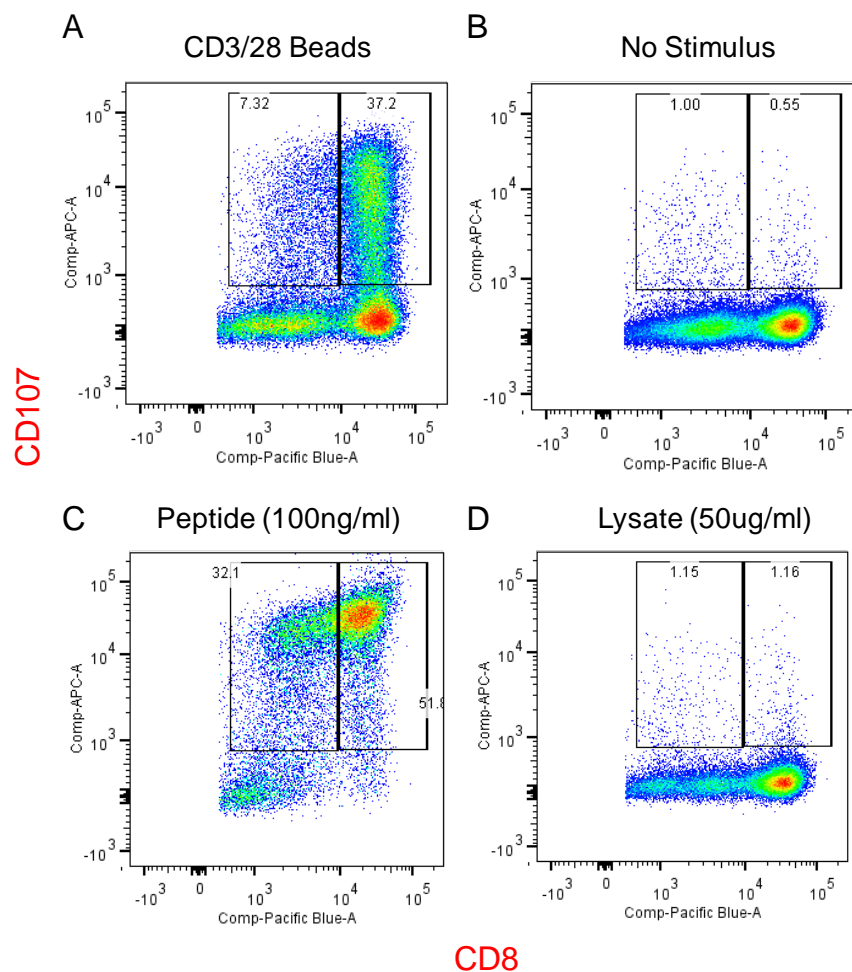
Figure 12. Reactivity of cancer specific T cell clones to antigen.
CD8$^+$ T cell clones were stimulated with positive controls CD3/CD28 beads (A) or target peptide (C), left unstimulated (B), or stimulated with HLA matched mDCs pulsed for 48 hours with the cancer cell line lysate (D). A 4 hour CD107 mobilization assay was performed and analyzed by flow cytometry.
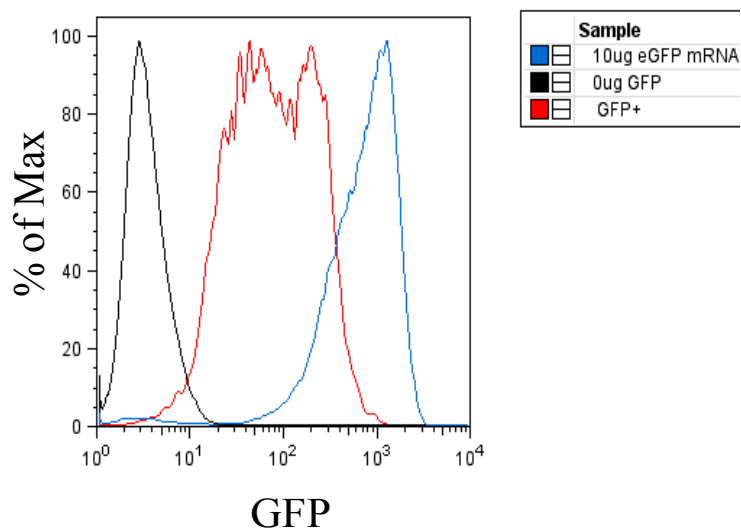
Figure 13. Electroporation of monocyte-derived dendritic cells
Immature mDCs were electroporated with 10ug eGFP mRNA (blue) or underwent a Mock transfection (black). HMY-A2-GFP expressing cell line was used as a positive fluorescent control.
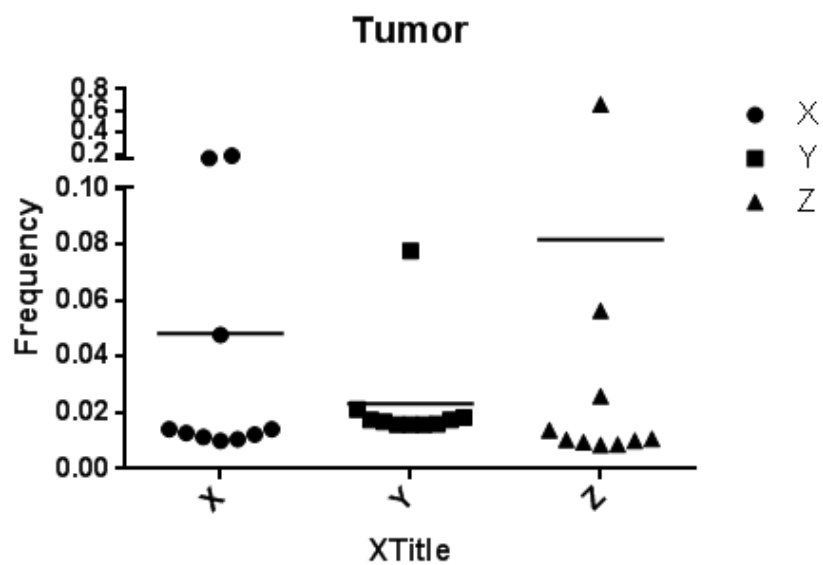
Figure 14. Frequencies of the most dominant T cell clones in tumor tissue.
Immune cells isolated from tumor samples were sequenced for their total TCR repertoire. Here we graph the frequencies of the 10 most dominant clones from the tumor samples of three different patients: X, Y, Z.